Combinatorial Chemistry

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# A Fragment-Based In Situ Combinatorial Approach To Identify High-Affinity Ligands for Unknown Binding Sites**

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*Dedicated to Professor Robert E. Ireland*

With his publication "On the attribution and additivity of binding energies" in 1981, Jencks[1] launched a new area in medicinal chemistry, that is, the so-called fragment-based drug discovery (FBDD).[2] When fragments are linked, their individual binding energies are additive. In addition, because of the reduction of translational and rigid body rotational degrees of freedom, the entropy barrier is markedly lowered.[3] Thus, by linking two low-affinity fragments, a new ligand with a substantially improved affinity for the target can be generated. However, this intriguing concept resulted in only a few scattered applications[4,5] and had no immediate impact on drug discovery. For a practical application of this strategy two problems remained to be solved; firstly, how suitable fragments that bind to proximal binding sites (so-called first- and second-site fragments) can be identified, and secondly, how these fragments can be linked without distortions of their individual binding modes.

The rapid development of this promising area[6] was initiated in 1996, when a conclusive practical demonstration of FBDD, called structure–affinity relationship by NMR (SAR-by-NMR) was reported.[7] With this novel approach, antagonists with nanomolar affinities were rapidly identified by tethering two fragments that were individually optimized by NMR spectroscopy. However, the implementation range of this technique was limited by the requirement for labeled proteins ($^{13}C$ and $^{15}N$) and for structural information on the binding site in order to design the linker. Subsequently, a broad array of innovative strategies for screening fragments were reported, for example, the needle approach[5] or tethering techniques detected by mass spectrometry.[8] Furthermore, the problem of the linker design was addressed by, for example, Sharpless and co-workers,[9] who used the target itself as an atomic-scale reaction vessel for creating its own inhibitor or by applying a shape-modulating linker design.[10]

Herein we present a novel fragment-based approach that does not require any spatial information on the binding site and can be conducted with modest amounts of unlabeled protein. Our target is the myelin-associated glycoprotein (MAG, Siglec-4), a sialic acid binding immunoglobulin-like lectin (Siglec),[11] which inhibits as one of several myelin components axonal regrowth after injury.[12] The recently reported use of monovalent glycosides[13] to reverse MAG-mediated blocking of axonal regeneration encouraged the search for high-affinity ligands. Oligo- and monosaccharide derivatives based on the ganglioside GQ1bα,[14] which was the hitherto best reported natural MAG antagonist, exhibit only micromolar affinities.[15] Therefore, an alternative approach to identify high-affinity ligands was required.

Because the crystal structure of MAG is not yet available, a homology model[16] based on the crystal structure of sialoadhesin (Siglec-1),[17] another member of the Siglec family, was investigated. This model revealed a shallow, unstructured binding site, which does not provide any obvious additional interaction sites and therefore little prospect for success by a structure-based approach. This result prompted us to develop a novel, three-step fragment-based in situ combinatorial approach, which is especially suited if little or no structural information on the binding site is available.[18] A first-site ligand with a moderate, that is, micromolar affinity, either based on a physiological ligand or identified by random screening, serves as starting point. In order to search for second-site ligands, members of a fragment library, which bind to the target protein, are identified by an NMR experiment that is based on the change of their transverse magnetization decay upon binding (Figure 1a).[19]

From these hits, fragments that bind to a second site located adjacent to the first site are identified by their enhanced paramagnetic relaxation caused by the spin-labeled first-site ligand (Figure 1b). Successful applications of spin labels for ligand screening[20a–c] as well as the characterization of binding sites[20d] have already been reported. Because the paramagnetic relaxation is distance-dependent, not only fragments that bind at a proximal subsite, but also their spatial orientation and hence the correct linking point can be elucidated.[20e] Finally, the target protein is incubated with a library of first- and second-site fragments functionalized with azido or acetylene groups at the end of flexible methylene chains of variable length (Figure 1c). Only in the case of an

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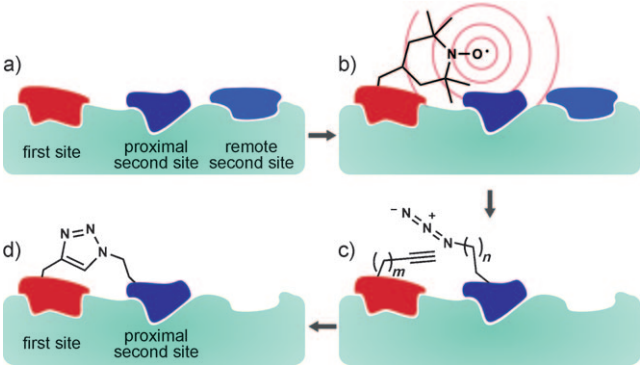



***Figure 1.*** Fragment-based in situ combinatorial approach for the identification of ligands for an unknown binding site. a) Identification of second-site ligands based on their transverse magnetization decay upon binding; b) selection of second-site ligands binding adjacent to the spin-labeled first-site ligand; c) incubation of the target protein with libraries of first- and second-site ligands; d) a high-affinity ligand is generated by receptor-mediated triazole formation. The receptor is shown in green.

optimal spatial orientation, which results from the binding of two compatible fragments to MAG, is a high-affinity ligand generated by a receptor-mediated triazole formation (Figure 1 d).[9]

For our investigations, recombinant MAG, which consists of the three N-terminal domains of MAG and the Fc fragment of human antibody immunoglobulin G (IgG; $MAG_{d1\text{-}3}$-Fc) was expressed in chinese hamster ovary (CHO) cells and affinity purified on protein A agarose.[21] As a first-site ligand, the sialic acid derivative **1** with 137 μM MAG affinity[15c] was selected (Figure 2).

Our fragment library for the identification of second-site ligands was composed of 80 low-molecular-weight ($M_w <$ 300), moderately lipophilic ($c\log P < 3$; $P =$ partition coefficient octanol/water) and highly soluble organic molecules. Whereas the limitations of $M_w$ and $\log P$ for fragments are well documented (Rule of Three),[22] water solubility is a prerequisite for the success of the planned NMR spectroscopic screens. The fragment library was divided into sublibraries, each of which contained 6 to 10 compounds. The sublibraries were composed according to two criteria: 1) the members of a sublibrary do not interact with each other and 2) each component shows at least one isolated resonance in the $^1$H NMR spectrum of the sublibrary.

Members of the sublibraries that bind to $MAG_{d1\text{-}3}$-Fc were identified based on the change of their transverse magnetization decay, which occurs upon complex formation.[23] The magnetization decay is molecular-weight-dependent and therefore allows a differentiation between small ligands, that is, fragments in solution, and large molecules, that is, fragments that are bound to the receptor. When the $^1$H NMR spectra of the sublibraries were recorded at relaxation times of 10 and 200 ms, the low-molecular-weight fragments of the sublibrary experienced only small magnetization decays. When $MAG_{d1\text{-}3}$-Fc was added, those fragments that bind to the protein, that is, those that formed a fragment–protein complex, behaved as large molecules and therefore suffered

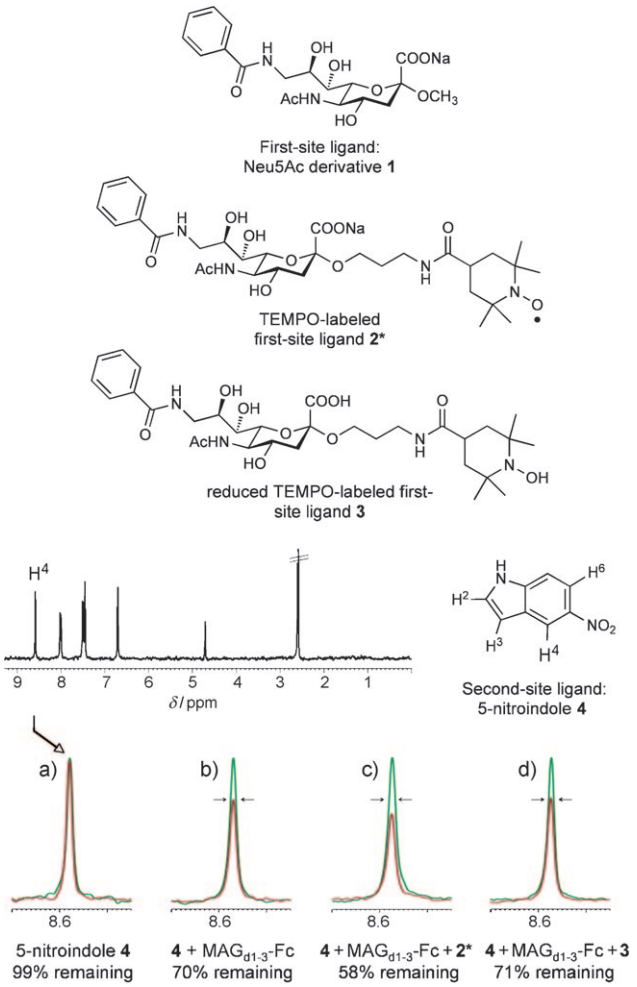



***Figure 2.*** Principle of second-site screening. a) $^1$H NMR resonance of H-C(4) of 5-nitroindole **4**; b) decay of transverse magnetization caused by binding of **4** to $MAG_{d1-3}$-Fc; c) paramagnetic relaxation enhancement on **4** caused by the spin-labeled first-site ligand **2***; d) recovery of the paramagnetic relaxation enhancement by the addition of ascorbic acid, which reduced **2*** to **3**.

from large magnetization decays. A number of fragments of the various sublibraries were identified to bind on the surface of the target protein. However, their exact binding site was still not known.

For the identification of the hits that bound adjacent to the binding site of **1**, spin–spin relaxation NMR experiments reported by Jahnke et al.[20a] were applied. Spin–spin relaxation rates are proportional to the product of the squares of the gyromagnetic ratio γ of the involved spin. Although it is the second highest among all isotopes, the γ value of protons and hence also the spin–spin relaxation rate are small. However, for the detection of ligand binding, the spin–spin relaxation rates should be as large as possible. Because the γ value of an unpaired electron is 658 times larger than that of a proton, the spin–spin relaxation rates caused by an unpaired electron on protons (known as paramagnetic relaxation enhancement) are dramatically larger than the effect of a nuclear–nuclear interaction.[24] Therefore, the spin-labeled first-site ligand **2***,

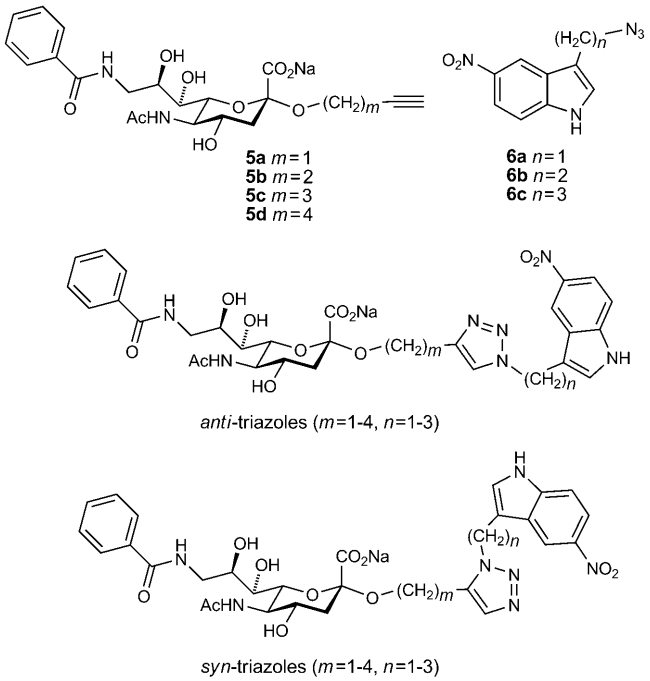
which contains an unpaired electron, was synthesized (Figure 2; for the synthesis see the Supporting Information). Compound **2*** is a conjugate of first-site ligand **1** and the TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl) spin label. Compound **1** and the *N*-hydroxy derivative **3**, which is the reduced form of TEMPO derivative **2***, exhibited similar affinities (**1**: 137 μM; **3**: 96 μM) in surface plasmon resonance (SPR) experiments[15c] as well as comparable transfer of magnetization from MAG in saturation transfer difference NMR (STD NMR).[25] It was therefore assumed that the TEMPO spin label does not substantially alter the binding mode of the first-site ligand.

The sequence of NMR experiments required for the identification of a second-site ligand binding adjacent to the first-site ligand is shown for the second-site ligand 5-nitroindole **4** in Figure 2. When the $^1$H NMR spectrum was recorded at two relaxation times, 10 and 200 ms, only a minimal decay of magnetization—as exemplified for H-C(4) of **4**—was observed (Figure 2a). Addition of $MAG_{d1\text{-}3}$-Fc led to a pronounced decay of transverse magnetization, thus indicating binding of **4** (Figure 2b). When the spin-labeled first-site ligand **2*** was added, a further enhancement of paramagnetic relaxation was observed, therefore suggesting simultaneous binding of **2*** and **4** at neighboring binding sites (Figure 2c).[20a] Finally, when the spin label was reduced by adding ascorbic acid to the NMR sample, the effect was cancelled, thus attributing the paramagnetic relaxation effect unambiguously to the spin label (Figure 2d).

Because the paramagnetic relaxation enhancement caused by the spin label is distance-dependent and is therefore potentially different for each proton of the second-site ligand,[24] the spatial orientation of **4** could be determined. The effect is largest for H-C(2), thus indicating that this proton is facing the spin label. Therefore, the carbon atoms of the pyrrole ring of 5-nitroindol **4** represent the optimal linking positions (see the Supporting Information).

For the linking of an appropriate first- and second-site ligand, the in situ click chemistry approach reported by Sharpless and co-workers[9] was applied. In this approach, the triazole formation does not result from copper catalysis but from the receptor-mediated preorganization of azide and acetylene to enable the [3+2] cycloaddition. For libraries of first- (**5a–d**) and second-site ligands (**6a–c**; Scheme 1, for synthetic details see the Supporting Information), two requirements had to be met. Firstly, flexible spacer arms should guarantee that the two terminal functionalities, that is, the acetylene and the azido groups attached to the first- and second-site ligand, can adopt the optimal spatial orientation to undergo a [3+2] Huisgen cycloaddition reaction.[26] Secondly, the entropic penalties associated with rotatable bonds should be kept low by the short spacers.[27] This requirement is in agreement with the reach of the spin label of about 10 Å.[24]

In previous in situ click chemistry studies,[9a–d] building blocks with nano- to low micromolar affinity for the target have been utilized. Because our ligands exhibit affinities in the range of 100 μM to millimolar as estimated by SPR[15c] and the fluorescent hapten binding assay,[21] their receptor-mediated linking represents a borderline case similar to that already discussed by Fokin et al.[9e]

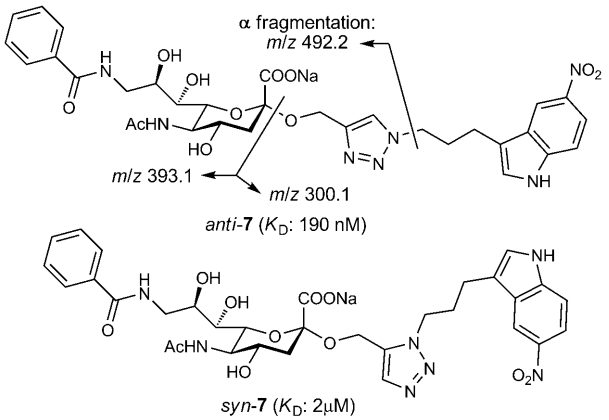



***Scheme 1.*** The library of first-site ligands consisted of four members, **5a–d**, the library of second-site ligands of three, **6a–c**. In total, 24 different triazoles, 12 *anti*-triazoles and 12 *syn*-triazoles could be formed.

For the in situ click experiment, $MAG_{d1-3}$-Fc[21] (6.47 μM) was incubated with a mixture of the four first-site ligands **5a–d** (each 330 μM) and the three second-site ligands **6a–c** (each 660 μM) in phosphate buffer (pH 7.4) at 37 °C for 3 days, thus in principle permitting the formation of 12 *syn*-substituted and 12 *anti*-substituted triazoles. The differing concentrations of the first- and second-site ligands were used to compensate for their different affinities for MAG (see above). The analysis of the supernatant by LC–MS in selected ion mode (SIM) after 3 days showed one major new molecular ion of *m*/*z* 694.2, which fits for three *syn*- and three *anti*-substituted triazoles, formed by [3+2]cycloaddition of **5a** and **6c**, **5b** and **6b**, and **5c** and **6a**, respectively. In addition, the other possible molecular ions were also present in low, but still detectable amounts. A control experiment carried out in the absence of MAG showed that the cycloaddition products were formed at comparable rates, which can be attributed to uncatalyzed background reactions.

The differentiation between the three *syn*/*anti* pairs with the same molecular weight but different spacer patterns was possible by using mass spectrometry (Scheme 2 and the Supporting Information). In the MS–MS mode with negative ionization, only two major fragments, with *m*/*z* 300.1 and *m*/*z* 393.1, could be detected beside the molecular ion (*m*/*z* 694.2). These species are formed by fragmentation of the glycosidic bond. Although the collision energy was varied from 5 to 50 V, fragments originating from exocyclic α fragmentation of the triazole could not be identified. However, a fragment *m*/*z* 492.2 was identified by SIM; this fragment can only be obtained by α fragmentation of the *syn*- and *anti*-triazoles **7** formed from **5a** and **6c** (see the Supporting




***Scheme 2.*** As differentiation between *anti*-**7** and *syn*-**7** was not possible by spectroscopic means, they were both synthesized (see the Supporting Information) and biologically evaluated. Differentiation can be unambiguously made between the three possible *syn*/*anti* pairs with a molecular weight of 694.2 according to the fragments obtained by α fragmentation.

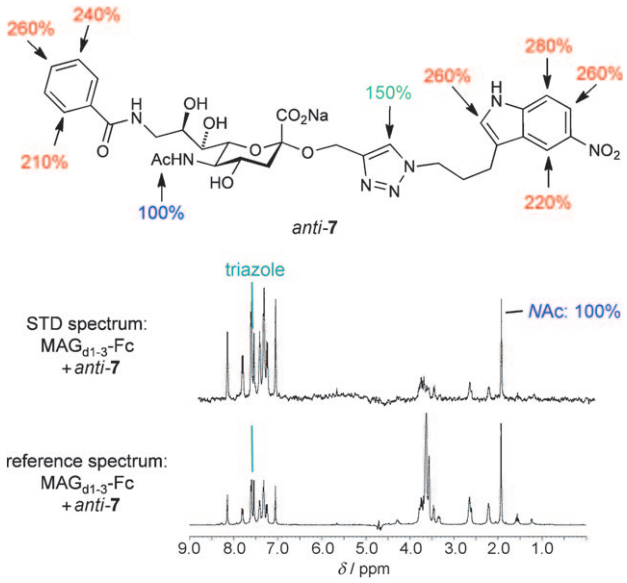



***Figure 3.*** The binding epitope of *anti*-**7** was mapped through STD NMR experiments. The percentages shown in the structure indicate the level of transfer of magnetization to a particular hydrogen atom relative to transfer of magnetization received by the *N*-acetyl methyl group. The large percentages on the benzamide hydrogens are consistent with previously reported SAR studies.[15d] In addition, large percentages were identified on the nitroindole, thus rationalizing the enhanced affinity of *anti*-**7**.

Information). As a further differentiation between the *syn* and *anti* product was not possible, *syn*-**7** and *anti*-**7** were synthesized under thermal conditions (see the Supporting Information) and biologically tested. In an SPR experiment with MAG$_{d1-3}$-Fc immobilized on a protein A modified chip surface,[15c] $K_D$ values of 190 nM for *anti*-**7** and 2 μM for *syn*-**7** were obtained (Scheme 2). This result leads to the assumption that *anti*-**7** was formed in the in situ click experiment, although the formation of both, *anti*-**7** and *syn*-**7** can not yet be excluded. An MAG antagonist that exhibits a 1000-fold improved affinity relative to the tetrasaccharide epitope of GQ1bα ($K_D$: 180 μM[15b]) was identified for *anti*-**7**.

STD NMR[25] experiments showed large contributions to binding from both terminal aromatic groups, the benzamide and the 5-nitroindole moiety (Figure 3). In addition, the triazole linking the first-site ligand **5a** and the second-site ligand **6c** also contributes to the interaction with MAG. This observation is consistent with previous experiments where the replacement of the methyl aglycon in sialoside **1** by a benzyl substituent led to an affinity enhancement that was approximately 10-fold.[15h]

In summary, the identification of a second-site ligand by NMR screening using a spin-labeled first-site ligand[20a] followed by a receptor-mediated linking of first- and second-site ligand[9] yielded a nanomolar MAG antagonist, which is currently under further biological evaluation. The synthetic effort is substantially reduced compared to a conventional approach. Beside the composition of the second-site library and the synthesis of the first-site ligand substituted with TEMPO, only seven compounds had to be synthesized to successfully identify a high-affinity antagonist. A particular strength of this approach is that no structural information of the target protein is required.

The identification of a nanomolar mimetic of the physiological ligand is extremely tedious, especially for the shallow binding sites of various lectins such as the sLe$^x$-E-selectin interaction. In many cases the approach is only partially successful, for example, for the trimannoside-DC-SIGN interaction.[15a] When applied to these proteins, the presented fragment-based in situ combinatorial approach could be valuable in supporting the identification of high-affinity glycomimetics and could develop into a useful tool in drug discovery.

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